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Increased expression of CD95-ligand and other apoptotic signaling factors by fumonisin B_1, a hepatotoxic mycotoxin, in livers of mice lacking tumor necrosis factor α

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Abstract

Fumonisin B_1 (FB_1), a mycotoxin, is a potent inhibitor of ceramide synthase, and produces organ-, species-, and even gender-specific toxic responses in animals. The hepatotoxic response of FB_1 in mice involves accumulation of free sphingoid bases and induction of inflammatory cytokines including tumor necrosis factor α (TNFα). The FB_1-induced hepatotoxic responses were reduced in mice lacking TNFα receptor (TNFR) 1 or TNFR2. However, the hepatotoxicity was exacerbated in mice lacking TNFα. We therefore investigated the modulation of various other apoptotic signaling factors in TNFα-knockout (TKO) mice compared to wild-type (WT) strain after repeated daily subcutaneous injections of 2.25 mg/kg FB_1 treatment for 5 days. Expression of various signaling genes in liver was evaluated by ribonuclease protection assay. Expression of *CD95-ligand* (*FasL*) was more than doubled in TKO animals after FB_1 whereas it was unaltered in the WT group. FB_1 did not alter *CD95* expression in either strain; however, expressions of *TRAIL*, and downstream signaling factors *FADD*, *TRADD*, and *caspase 8* were higher in FB_1-treated TKO mice than in the corresponding WT animals. The TKO strain had a higher constitutive expression of apoptotic factors except *CD95L*. In addition to the CD95 and TNFα systems, the expression of apoptotic molecules *bcl-2*, *b-myc*, *c-myc*, *bax*, *max*, *mad* and *IL1α* was induced by FB_1 in TKO mice to a greater extent than in WT animals; many of these factors also had a higher constitutive expression in TKO animals than WT mice. Results indicated that FB_1 can induce CD95 modulated signaling when TNFα is absent. Differential constitutive expression of apoptotic genes in TKO mice may explain their increased sensitivity to FB_1. These results are important in characterizing the modulating effect of TNFα on apoptotic signaling and in explaining the unexpected sensitivity of mice lacking this cytokine in response to hepatotoxic xenobiotics.

Keywords: Tumor necrosis factor α; CD95; CD95L; TNF-knockout; Apoptosis; Fumonisin

The role of inflammatory cytokines in the initiation and development of liver toxicity has been well established. Liver-associated lymphocytes and their cytokine products, especially tumor necrosis factor α (TNFα) are involved in liver injury during alcoholic hepatitis [1]. Neutralizing antibodies to TNFα or interleukin (IL)-1 reduced the hepatotoxic response of acetaminophen in mice [2]. The involvement of TNFα was reported in hepatotoxicity induced by carbon tetrachloride, dimethylnitrosamine or cadmium [3–5]. TNFα and interferon γ (IFNγ) are involved in concanavalin A-induced hepatitis and in liver toxicity in response to repeated challenges of lipopolysaccharide [6,7].

Fumonisin B_1 (FB_1) is the most abundant of the toxic fumonisins. It causes liver toxicity characterized by apoptosis in mice [8]. This mycotoxin is one of several fumonisins produced by *Fusarium verticillioides*, and is a common contaminant in corn and corn products. FB_1 produces tissue- and species-specific damage in different

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animals. In horses FB_1 causes leukoencephalomalacia, in swine pulmonary edema, and in most species, including rodents, liver and kidney damage [9]. In mice and rats, gender-specific differences have been observed. FB_1 is a more potent hepatotoxin in female than in male mice and a more potent kidney toxin in male rats, compared to female rats or mice of either sex [10]. FB_1 has been implicated in esophageal cancer and primary liver cancer in people [9–11].

Fumonisins are structurally related to the free sphingoid bases, sphinganine and sphingosine, and thereby inhibit their incorporation into ceramide by inhibiting sphinganine- or sphingosine-*N*-acyltransferase (ceramide synthase). This is a critical mechanistic step that leads to disrupted cell function. Although the sensitivity of different tissues to the toxic effects of this mycotoxin differs, the inhibition of ceramide synthase by FB_1 and resulting accumulation of sphinganine have been reported in most tissues. For example, mouse kidney exhibited high accumulation of sphinganine in response to FB_1, yet it is not as sensitive as mouse liver to FB_1 toxicity as judged by histopathological criteria [8,12].

A variety of biochemical changes subsequent to the inhibition of ceramide synthesis and accumulation of free sphingoid bases in response to FB_1 have been reported [13]. These effects include modulation of protein kinase C and mitogen-activated protein kinases. We recently reported the induction of proinflammatory cytokines, especially TNFα, IFNγ and IL-12 in mouse liver after exposure to a single or repeated doses of FB_1 [14–16]. Macrophages isolated from FB_1-treated mice secreted more TNFα in response to lipopolysaccharide activation in vitro compared to the saline-treated control [17]. TNFα was also induced in macrophages directly treated with FB_1 and acute hematologic effects of FB_1 were antagonized by pretreatment of mice with anti-TNFα antibodies [17].

To further investigate the role of TNFα in FB_1-induced hepatotoxicity we have employed various transgenic strains of mice. In mice, hepatotoxicity to FB_1 repeated exposure was reduced in strains lacking TNFα receptor (TNFR) 1 or TNFR2 [18,19]. However, in mouse strain with deleted TNFα gene (TNF knockout (TKO) mice), the hepatotoxic response to FB_1 was not ameliorated despite the overwhelming evidence that TNFα is involved in the toxic effects of fumonisin [20]. We wanted to investigate in the current study the mechanism describing why the fumonisin hepatotoxicity was not reduced in the absence of TNFα pathways. Since FB_1 modulates several inflammatory cytokines and apoptotic signaling molecules in sensitive tissues, we hypothesized that in the absence of TNFα FB_1 modulates other apoptotic pathways including the CD95 (Fas) signaling in TKO. Subacute exposure of male BALB/c mice with FB_1 produced no changes in the CD95 signaling system including the expressions of *CD95*, *CD95-ligand* (*CD95L* or *FasL*), *Fas-associated death domain* (*FADD*), and *Fas-associated protein factor* (*FAF*) [15]. However, in the current study the CD95 system was markedly activated in mouse-strain lacking TNFα gene and can be responsible for the increased hepatotoxicity of these animals after treatment with FB_1. We hereby report the increased expression of *CD95L* and downstream molecules in the CD95 signaling along with the increased levels of *TNFα-related apoptosis-inducing ligand* (*TRAIL*) by FB_1 in TKO animals and discuss the importance of hepatic lymphocytes in producing hepatotoxicity.

1. Results

The increased hepatotoxicity in TNFα-null mice (TKO) as compared to wild-type (WT) mice in response to FB_1 has been reported elsewhere [20]. Increases of relative liver and spleen weights and peripheral leukocyte counts were noticed after FB_1 treatment in TKO strain only. The effects of FB_1 in the TKO strain were characterized by relatively higher levels of plasma alanine aminotransferase (4.7-fold greater in FB_1-treated TKO than the corresponding WT strain) and aspartate aminotransferase (2.5 times greater in FB_1-treated TKO animals than in FB_1-treated WT mice), and increased severity of microscopic liver lesions over the corresponding WT strain [20]. In the current report, we have further characterized the incidence of apoptotic cells using terminal uridine nucleotide end labeling (TUNEL) and proliferating cells by immunohistochemistry for proliferating cell nuclear antigen (PCNA). The illustration of TUNEL-positive cells in mouse liver is presented in Fig. 1. These results were in excellent agreement with those obtained by examination of hematoxylin and eosin stained sections and those reported previously [20]. Very few apoptotic cells were noticed in the liver of one saline-treated mouse (others showed none), whereas there was a considerable number of TUNEL-positive cells in livers of both WT and TKO mice after FB_1 treatment. Because of a high variability among mice of the same group the differences between FB_1-treated WT and TKO were not statistically significant, although a greater incidence of TUNEL-positive cells in livers from TKO mice was apparent. It was therefore clear that the lack of TNFα had no protective effect against FB_1 hepatotoxicity in the TKO mice [20].

The incidence of PCNA-positive cells in livers from different groups of animals is shown in Fig. 2. Livers from saline-treated WT mice had a few scattered proliferating cells. Significantly more PCNA-positive cells were found in the livers of the FB_1-treated WT mice. The number of PCNA-positive cells in liver was increased by FB_1 treatment in the TKO strain to a similar or slightly greater extent than in WT mice.

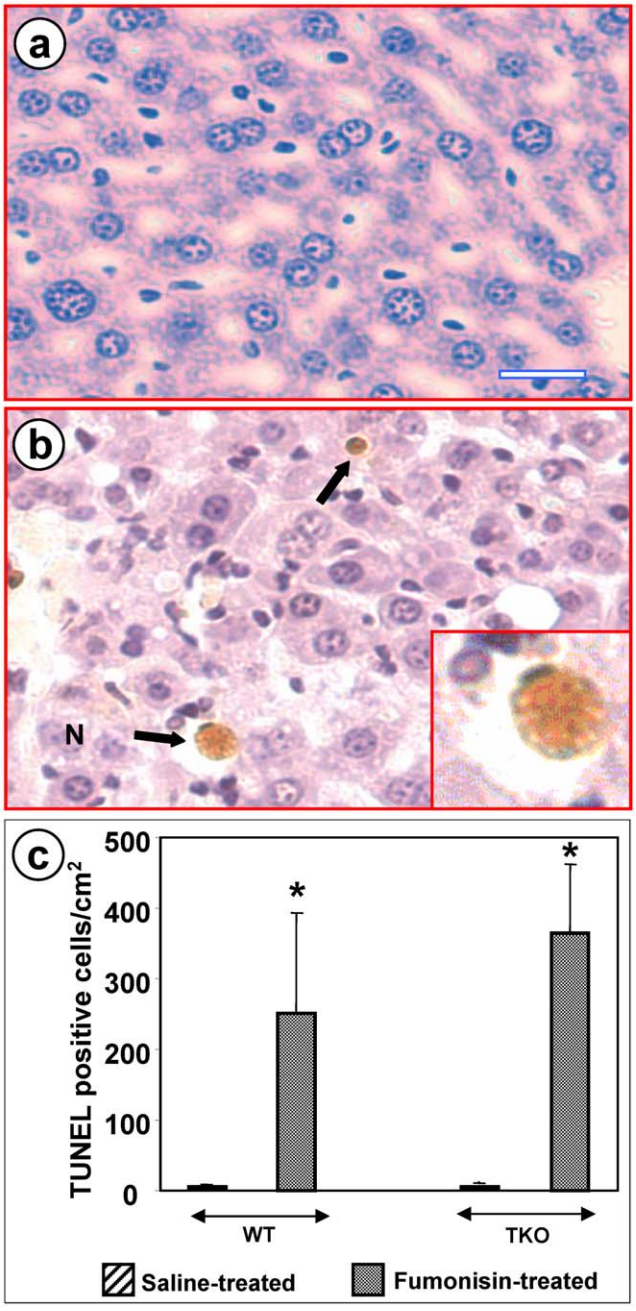

Fig. 1. Enumeration of apoptotic cells in liver by TUNEL assay. (a) Liver from saline-treated WT mouse showing normal architecture and no TUNEL-stained cell. Bar in the lower right equals 40 μm. (b) Liver from FB_1-treated TKO mouse; arrows indicate some of the TUNEL-positive cells. Cells are irregular with less pronounced nuclear morphology, the detailed pathological description of hematoxylin–eosin stained sections has been presented elsewhere [20]. Arrows indicate apoptotic cells. The inset represents a single TUNEL-positive nucleus (N) separated from the surrounding cytoplasm. (c) The number of TUNEL-positive cells in tissue sections normalized to unit area (mean ± standard error, $n = 5$). The symbol (*) indicates a significant difference from the saline-treated group of the same strain ($p < 0.05$).

The expression of various genes involved in apoptosis and cell cycling was investigated by the ribonuclease protection assay (RPA). This technique is quantitative and the phospho-imaging signal is linear within several orders of magnitude. Representatives of selected RPA

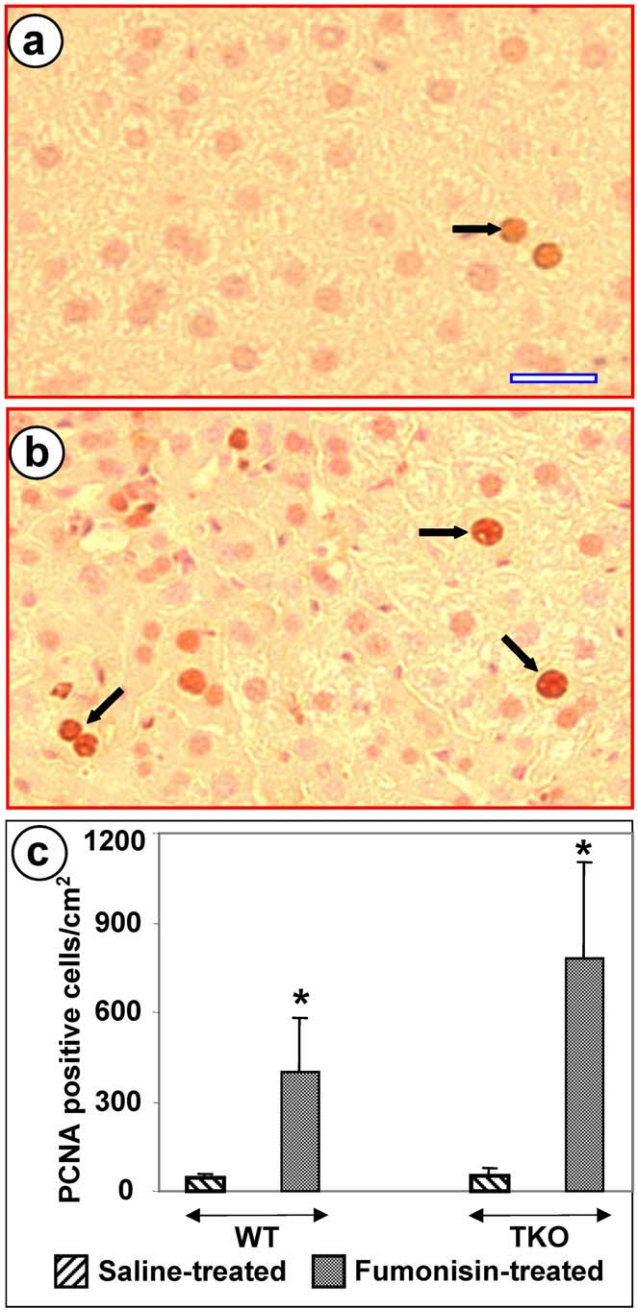

Fig. 2. Enumeration of cells in liver showing the expression of PCNA by immunohistochemistry. (a) Liver from saline-treated WT mouse showing normal architecture and two PCNA-positive cells (one marked by an arrow). Bar in the lower right equals 40 μm. (b) Liver from FB_1-treated TKO mouse; arrows indicate some of the PCNA antibody-stained cells. (c) The number of PCNA-positive cells in tissue sections normalized to unit area (mean ± standard error, $n = 5$). The symbol (*) indicates a significant difference from the saline-treated group of the same strain ($p < 0.05$).

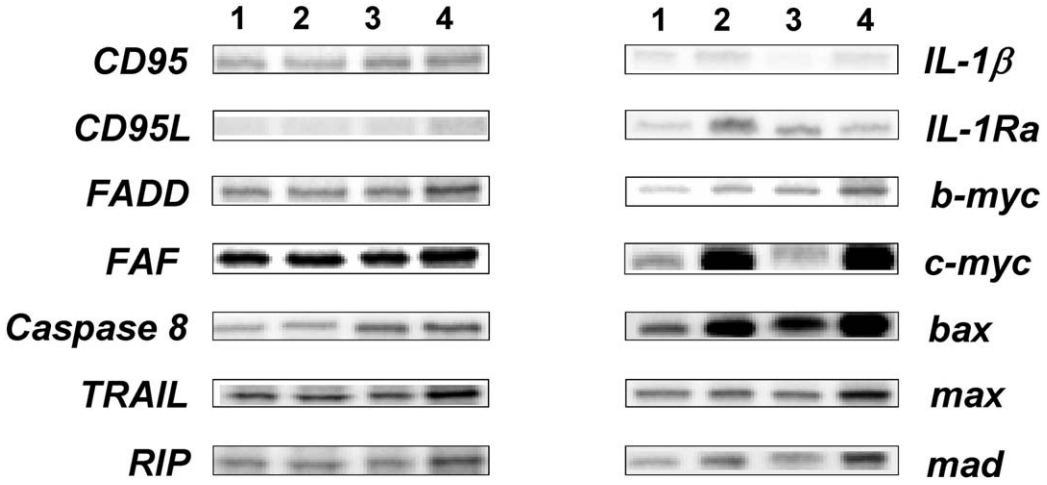

Fig. 3. Representative gels from the RPA assay for the expression of selected genes. The respective lanes indicate sample from: 1, a saline-treated WT mouse; 2, FB_1-treated WT mouse; 3, saline-treated TKO mouse; and 4, FB_1-treated TKO mouse.

bands are illustrated in Fig. 3. The RPA was conducted using RNA extracted from livers of individual mice from groups of five animals each and normalized to the expression of *L-32*, a ribosomal protein as an internal standard, for each individual sample.

Fig. 4 illustrates the data from various groups of mice regarding the expression of CD95-related molecules. In the TKO mice, the constitutive expression of *CD95* was nearly doubled, although the treatment with FB_1 did not alter the *CD95* expression in either strain of the mice. However, the expression of *CD95L* (*FasL*) was increased nearly 2.3 times by FB_1 treatment in the livers from TKO mice, while it was unaffected in the WT group. Expression of downstream CD95-related factors, such as *FADD*, *Fas-associated phosphatase* (*FAP*) and *FAF*, tended to be higher in FB_1-treated TKO animals than in FB_1-treated WT mice (Table 1). This was also true for *caspase 8*, and other caspases examined (*caspase 3* and *caspase 6*; data not illustrated since differences were not statistically significant between FB_1 and saline-treated groups in either strain).

The expression of other apoptotic signaling and interacting molecules is also presented in Fig. 4. It is interesting that the expression of *TRAIL* was increased by FB_1 in both WT and TKO mice; but because the constitutive expression of *TRAIL* was higher in TKO mice, higher amounts of *TRAIL* were present in TKO than in the WT strain after FB_1 treatment. The expressions of *TNFR1*, *TNFR-associated death domain* (*TRADD*), and *receptor interacting protein* (*RIP*) were all increased in a similar way; the increased level in TKO mice after FB_1 was greater than those for the corresponding WT group.

Expression of selected cytokines and related molecules is also provided in Fig. 5. FB_1 induced *IL-1α* in the liver of TKO mice only, whereas the expression of *IL-1β* was not significantly altered in either strain. The expression of *IL-1 receptor antagonist* (*IL-1Ra*) was increased by FB_1 in WT mice only and the levels of *IL-18* were not significantly affected by FB_1 in either strain.

Fig. 5 also presents the expression of other selected apoptotic factors measured by RPA. Levels of expression for *max*, *mad* and *b-myc* were increased by FB_1 in TKO mice only; those for *bax* and *c-myc* were increased by FB_1 in both strains of mice employed here. Again, the extent of increase for *bax* and *c-myc* was higher after FB_1 treatment in TKO animals than the respective WT group. The expression of *bcl-2* was not altered by FB_1 treatment of animals in either strain.

An observation of interest in this study was the finding that the constitutive expression of a variety of apoptotic factors was higher in TKO than the respective WT mice. Treatment of these animals with FB_1 further increased these molecules; the expression was increased to a greater extent in FB_1-treated TKO mice than the FB_1-treated WT animals (Figs. 4 and 5). Of particular interest here are *CD95L*, *bax*, *max* and *b-myc*, all of which were induced greater than 2-fold in FB_1-treated TKO than in the FB_1-treated WT mice.

2. Discussion

Results presented here supported our hypothesis that signaling pathways other than TNFα can be activated to a greater extent by FB_1 in the absence of TNFα. In particular, the expression of *CD95L* was increased by FB_1 only in the animals without TNFα and that of *TRAIL* was induced in the TKO mice to a greater extent than in WT animals. FB_1 did not induce *CD95* or *CD95L* in intact WT strain employed here; a similar finding was observed earlier for BALB/c mice [15]. The BALB/c animals also had no increase in *FADD*, *FAF*, *RIP* or *TRAIL* [15], which were significantly elevated by FB_1 in the present

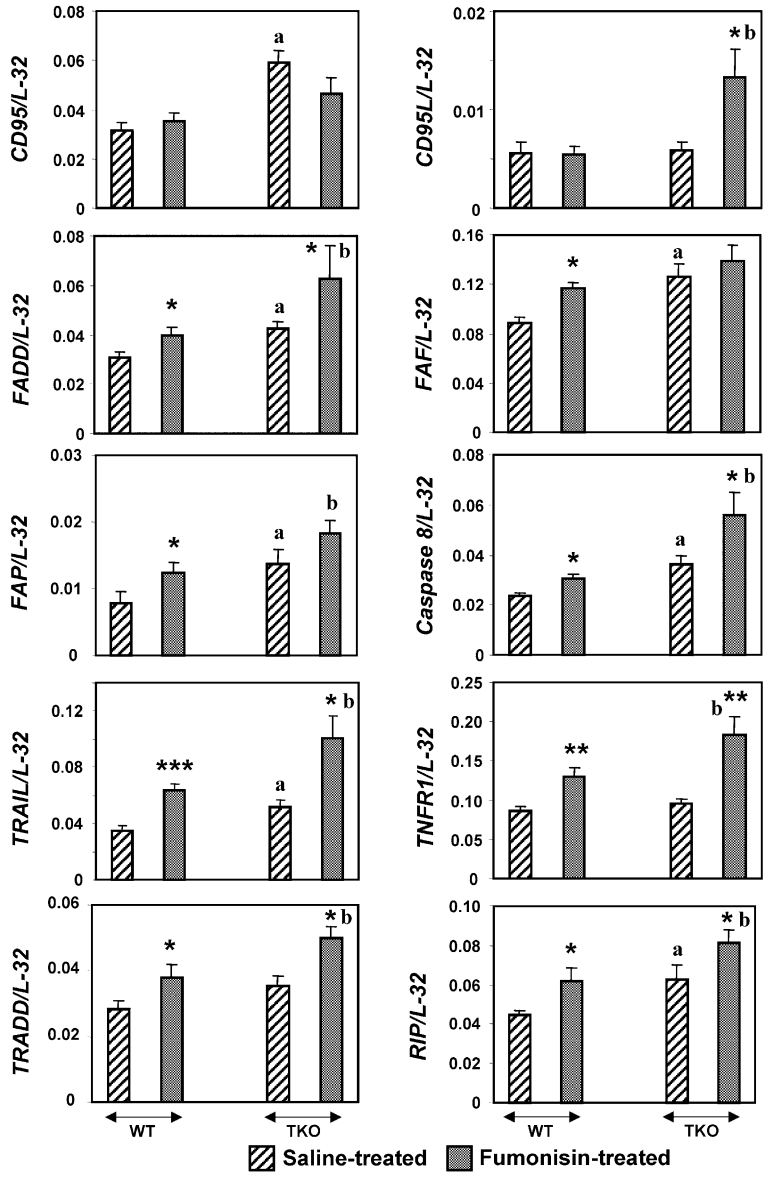

Fig. 4. The fumonisin B_1-induced changes in the mRNA expression for different genes related to CD95 and TNFα receptor pathways. The hepatic RNA from an individual animal was subjected to RPA and normalized against *L-32*. Mean ± standard error ($n = 5$). Asterisks on bars indicate a significant difference from the saline-treated group of the same strain at $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$. Comparisons between the two strains with similar treatment are indicated as: a, significantly different from the saline-treated WT ($p<0.05$); b, significantly different from the FB_1-treated WT ($p<0.05$).

WT (B6,129) strain. In the absence of TNFα the TKO mice had more than twice the *CD95L* after FB_1 treatment than saline-treated TKO or WT animals.

The role of cytokines in toxicant-induced hepatic injury has been established. Cytokines differentiate naive T helper (Th) cells into Th1 and Th2 phenotypes. The imbalance between Th1 and Th2 cells has been related to pathological processes [21]. Liver-associated T lymphocytes spontaneously secrete IL-1α, lL-6 and TNFα in cultures [1,22]. It has been suggested that TNFα can cause liver cell apoptosis through CD95 expressed by liver cells [1]. When TNFα is not produced, as in the TKO mice, other factors may likely compensate for its absence. In the current study we have also shown a significant increase in the induction of *IL-1α* in TKO mice after FB_1 exposure compared to intact WT animals.

The effect of TNFα is mediated mainly via its two receptors, TNFR1 and TNFR2 [23]. In the current study and also in a previous study from our laboratory [15] we observed that FB_1 increased the expression of *TNFR1*. The effect of FB_1 on *TNFR2* expression has not been investigated. Although the TKO mice were devoid of *TNFα* expression, it is possible that FB_1 may have effects on other signal elicitors such as lymphotoxin α (also referred as TNFβ). It has been reported that lymphotoxin α is capable of binding both TNFR1 and TNFR2 [24]. In

Table 1
Relative abundance of mRNA in the livers of WT and TKO after saline or fumonisin B_1 treatment

Gene name	GeneBank accession #	% Change in TKO over WT[a]		Description
		Saline-treated	FB_1-treated	
CD95L	U06948	6.8	148.8*	Apoptosis inducing ligand
CD95	M83649	89.1*	31.0	Apoptosis inducing receptor
FADD	NM_010175	37.2*	58.2*	Fas associated death domain
FAF	XM_127359	42.7*	19.6	Fas associated protein factor
FAP	D83966	73.4*	48.0*	Fas associate protease 1
TRAIL	U37522	46.3*	58.4*	TNFα related apoptosis inducing ligand
TRADD	AA013699	26.0	32.1*	TNFR associated death domain
RIP	U25995	39.9*	30.5*	TNFR interacting protein
TNFR1	M59378	11.2	40.7*	P55 TNFα receptor
Caspase 3	U49929	15.7	38.7	Apoptotic protease
Caspase 6	NM009811	31.4	42.8	Apoptotic protease
Caspase 8	AF067834	55.3*	83.9*	Apoptotic protease
IL-1α	NM_010554	53.8*	76.6*	Inflammatory cytokine
IL-1β	M15131	−34.5	−36.3	Inflammatory cytokine
IL-1Ra	M57525	32.4*	−25.2	IL-1 receptor antagonist
IL-18	D49949	−5.6	−12.8	IFNγ-inducing cytokine
MIF	BC024895	13.3	−13.7	Macrophage migration inhibitory factor
bad	L37296	−8.6	30.6	Apoptotic factor
bax	L22472	56.1*	159.0*	Apoptotic factor
mad	L38926	12.7	68.4*	Apoptotic factor
max	M63903	16.6	104.2*	Apoptotic factor
b-myc	W18410	42.0	127.7*	Oncogene, apoptosis
c-myc	X00195	129.4*	52.0*	Oncogene, apoptosis
bcl-2	NM_009741	34.7*	19.9	Anti-apoptotic factor

[a] The saline treated groups in TKO mice compared to WT mice (constitutive expression in the two strains), and FB_1-treated TKO mice compared to FB_1-treated WT mice, respectively.

* Significant change over the WT group receiving the same treatment ($p < 0.05$).

a preliminary study using the microarray technique we have observed an induction of *lymphotoxin α* in intact BALB/c mice along with an increase in *TNFα* (unpublished data). An increase in *lymphotoxin α* expression may contribute to hepatotoxicity, although its relative role after FB_1 treatment has not been determined. Our future studies will investigate the role lymphotoxin α may play in FB_1-induced hepatotoxicity in mice.

It should be stated here that TKO mice were different in their expression of various apoptotic signaling than the normal intact mice. Furthermore, while these mutants are viable and fertile, they completely lack splenic B cell follicles and cannot form organized follicular dendritic cell networks and germinal centers [25]. The TKO animals were relatively resistant to smaller doses of lipopolysaccharide after D-galactosamine sensitization but were sensitive to lethal doses of lipopolysaccharide. The organogenesis in Peyer's patch is normal in these mice but formation of B lymphocyte follicles is defective [26]. The TKO mice showed little or no initial response to heat-killed *Corynebacterium parvum* challenge, but developed a delayed vigorous inflammatory response leading to death [27]. A virulent mycobacterium did not induce granulomas in TKO mice [28], suggesting a differential response to antigenic challenge or an external stimulus in the absence of TNFα. As is apparent from the data in present study, the profile of a number of apoptotic signaling molecules is quite different in TKO mice than the WT animals.

Both CD95L and TRAIL have similar apoptotic mechanisms in tissues although they act via different receptors [29–31]. There is limited information on the expression of *CD95L* and *CD95* in mouse liver. Mouse liver possesses both CD95L and CD95 during development but its expression in adult mouse is limited to immune privileged tissues [32]. CD95-mediated apoptosis in cultured hepatocytes has been reported [33], and also in the adult mouse liver [22]. The distribution of CD95 was also shown in other types of cells; it was expressed in human epithelial cells with rare expression in mesenchymal cells [34]. The overexpression of CD95L may increase CD95L–CD95 signaling in the liver of FB_1-treated TKO mice vs. those of the WT strain.

The site of CD95L production in liver is not known at present. However, liver possesses a large number of resident lymphocytes. The number of lymphocytes in the liver is estimated at 10–20 million cells per gram tissue, which in the total organ equals nearly 15–20% of all lymphoid cells in the spleen [1]. The liver lymphocytes contain a high frequency of natural killer (NK) cells [35],

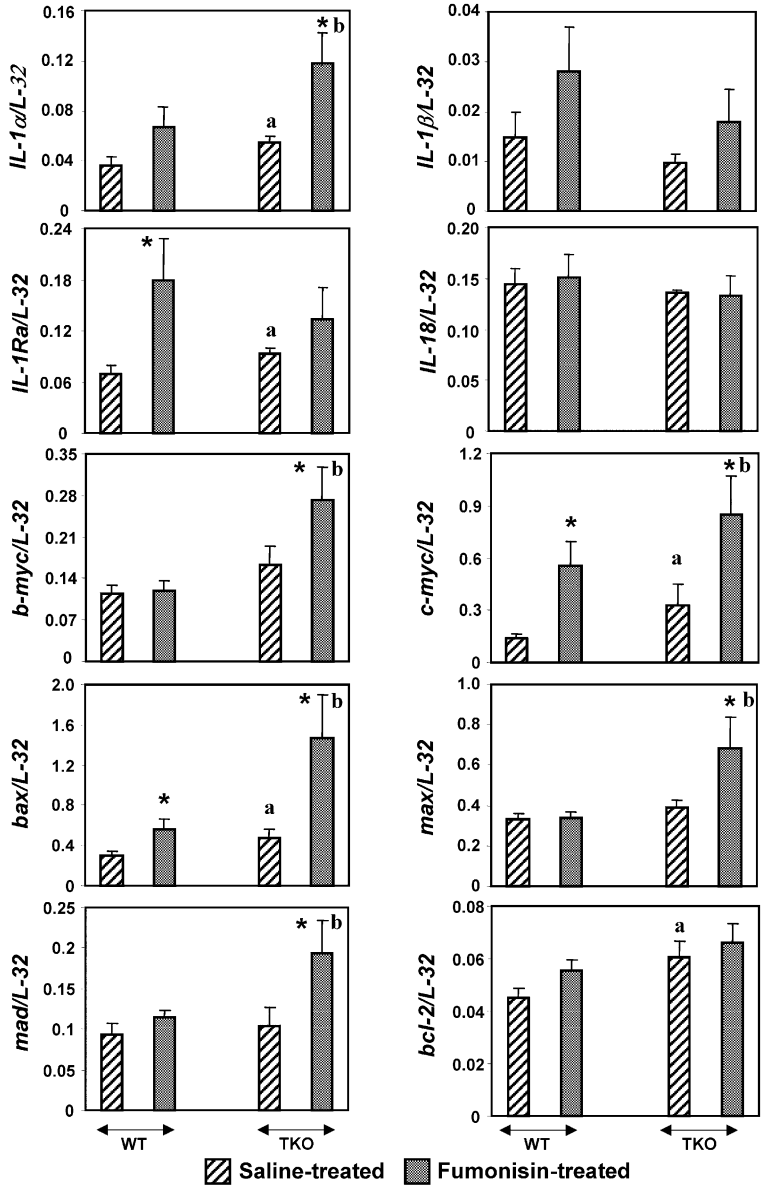

Fig. 5. The fumonisin B_1-induced changes in the mRNA expression for selected cytokines and apoptotic genes. The hepatic RNA from an individual animal was subjected to RPA and normalized against *L-32*. Mean ± standard error ($n = 5$). The symbol (*) indicates a significant difference from the saline-treated group of the same strain at $p < 0.05$. Comparisons between the two strains with similar treatment are indicated as: a, significantly different from the saline-treated WT ($p < 0.05$); b, significantly different from the FB_1-treated WT ($p < 0.05$).

and a high ratio of CD8+ cells compared to CD4+ cells. These lymphocytes are likely source of *CD95L* and also the T cell-related inflammatory cytokines, and have been implicated in alcohol-related liver disease [1]. Involvement of CD95 and its ligand in liver damage has been reported [22]. In the present study an increased expression of the cytotoxic molecules, including *CD95L*, by FB_1 may be an important determinant of hepatic injury in mice lacking TNFα.

The contribution of TRAIL to FB_1-induced liver injury can also not be underestimated. The TRAIL-induced apoptosis involves RIP [36], which is important given the increased constitutive expression of *RIP* in the TKO mice in the present study. The cytotoxic ligand *TRAIL* was induced by FB_1 in both strains used here; however, its overall expression after FB_1 in TKO mice was relatively greater than the correspondingly treated WT mice. The relative involvement of this ligand in hepatotoxicity has been emphasized [37], and a distinct receptor for TRAIL with associated death-domain has been cloned from mouse tissues [38].

The role of other signaling factors that participate in the apoptotic process can also not be ruled out. In the TKO mice many of these were present in higher levels after FB_1 treatment than in the WT animals. Overexpression of *FADD* and *RIP* is known to cause apoptosis and these factors also participate in the CD95 signaling mechanisms [39]. At the same time the

expression of other apoptotic molecules such as *max*, *mad* and *b-myc* was induced by FB_1 in TKO mice liver only. The increase in *bax* and *c-myc* was greater in TKO mice than in WT animals after FB_1 exposure. The role of these factors in apoptosis is well known [40–42]. Overexpression of these molecules will contribute to increased hepatotoxicity in TKO mice liver; the relationship of their increased expression with increased *CD95L* expression is not well understood and it is likely that the induction of various members of myc/max/mad family was due to an increased CD95 signaling.

It needs to be emphasized that the overall level of expression for an apoptotic factor rather than the increase by fumonisin may determine the ultimate outcome of its toxicity. For example in the case of TKO mice the constitutive expression of *CD95* in liver was 89% greater than in WT animals, the level was still higher after FB_1 treatment, and therefore may contribute to increased hepatotoxicity. We earlier reported that female mice are more susceptible to fumonisin hepatotoxicity than the males despite the fact that the magnitude of induction of various inflammatory cytokines was less than that in males but the constitutive levels of their expression were higher [14].

The induction of anti-apoptotic factors was somewhat less apparent in TKO animals than in WT mice. FAP-1, a protease factor, has been reported to inhibit the CD95-mediated apoptosis [43]. This factor was increased by FB_1 in WT mice liver but the change in TKO mice after FB_1 was not significant. The constitutive expression of *FAP* was greater in TKO mice than in WT mice; the relevance of this finding is not understood at the present time. Similarly, the expression of *IL-1Ra*, which inhibits the toxic responses of IL-1 cytokine, was induced by FB_1 in WT but not in the TKO mice. Lack of such inhibition will also help the enhanced hepatotoxicity of FB_1 in TKO animals [44]. Another factor involved in the protection from apoptosis is bcl-2. The expression of *bcl-2* was unchanged both in WT and TKO strains.

It is possible that the elevation of *CD95L* and other apoptotic factors is related to the disruption of sphingolipid metabolism by FB_1. We earlier observed that the level of sphinganine was increased 37-fold in TKO mice after FB_1 exposure vs. 12-fold in WT mice, compared to their saline-treated counterparts [20]. Similarly, sphingosine accumulated to nearly 2-fold of saline-treated controls in TKO mice after FB_1 exposure whereas its level remained unchanged in WT mice after a similar treatment. It is possible that an increased accumulation of sphinganine, sphingosine, or both, by FB_1 caused the induction of *CD95L* in TKO mice, and may also be responsible for the super-induction of apoptotic factors like *bax*, *max*, *mad* and *b-myc* in this strain. Free sphingoid bases like sphingosine and also its respective phosphate metabolite are known inducers of apoptosis in various cell models [45]. However, the pathways by which the signal from increased sphingoid bases or their phosphates ultimately leads to cellular death are not clearly elaborated. In intact mice the induction of TNFα and subsequently of other cytokines is a likely factor; however, the damage can be elicited even in the absence of TNFα pathway. The role of CD95 signaling indicated in this report may be one such alternate mechanism.

Effect of fumonisin on various cell cycling genes has been described earlier [46]. Rats treated with fumonisin showed higher levels of cyclin D1, p27 and PCNA in their liver. The changes were observed both in mRNA and protein expression. The involvement of TNFα pathways in fumonisin toxicity in CV-1 (African green monkey kidney fibroblasts) cells has been demonstrated [47]. The effects of fumonisin in these cells included cleavage of caspase 8 and the cytotoxic effect was abrogated by the baculovirus gene, an inhibitor of caspases, thereby suggesting the involvement of TNFα. Bcl-2 was not an effective inhibitor in this study.

Considering all the alterations produced by FB_1 in the expression of various apoptotic factors, it is evident that in TKO mice, FB_1 caused a markedly greater increase in *CD95L* and *TRAIL*, together with *IL-1α* and downstream signaling molecules like *FADD*, *TRADD* and *RIP*, than in the WT strain. A differential expression of these molecules in the absence of TNFα may account for the lack of resistance against FB_1 hepatotoxicity in TKO mice, and perhaps to an enhanced response in this strain. This increased induction was accompanied by a less pronounced increase of anti-apoptotic factors like *IL-1Ra* and *FAP* in TKO mice after treatment with FB_1. A differential expression of signaling factors in the liver of TKO mice and a variable response to FB_1 treatment in their expression may explain the lack of protection from hepatotoxic effects of FB_1 in this strain.

3. Materials and methods

3.1. Animals and housing

The transgenic strain derived from B6,129SF2 mice was procured from Jackson Laboratories (Bar Harbor, ME). The male TKO mice (B6,129S-Tnf^{tnf1Gkl}) were developed by Dr. G. Kollias of Hellenic Pasteur Institute (Athens, Greece) [25]. The animals were homozygous/homozygous male offsprings developed by backcrossing of several generations of knockout animals. The TKO mice were phenotypically identical to their counterpart B6,129SF2, hereafter referred to as the WT strain, procured from the same source. The knockout status of TKO animals was confirmed by polymerase chain reaction (PCR) on the genomic DNA from a small number of blood cells and hepatic cDNA. All animals were six weeks old upon arrival and were acclimated for one week before dosing. Mice were

group-housed in filter-barrier top cages under controlled environmental conditions, at 23 °C and 50% relative humidity with a 12-h light/dark cycle. Animals were provided free access to fumonisin-free food and tap water. Protocols for animal use followed the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

3.2. Treatment and sampling

Fumonisin (98% purity) was extracted from *F. verticillioides* culture material as described previously [48]. Its purity was assessed by comparing the FB_1 to multiple analytical standards using liquid chromatography–mass spectrometry (LC–MS) methods (R.D. Plattner, personal communication). Groups of five animals each were treated with five daily subcutaneous injections (1 ml/100 g body weight) of FB_1 in phosphate buffered saline (PBS), the dose per mouse being 0 (saline-treated controls) or 2.25 mg FB_1/kg/day. The dose and duration of treatment was selected based on our previous studies in which it produced a consistent and appreciable hepatotoxic damage in mice [14–20]. One day after the fifth and the final injection, the animals were killed with halothane. Liver samples were fixed in neutral formalin. Additional liver samples were quickly frozen on dry ice and stored at −85 °C until analyzed.

3.3. Determination of apoptosis in liver

Liver samples were fixed in 10% neutral-buffered formalin overnight and routinely paraffinized. Sections (5 μm) were prepared and subjected to TUNEL assay of apoptotic cells with a peroxidase-based Apoptag® Plus kit (Oncor Inc, Gaithersburg, MD). Briefly, sections were deparaffinated with two changes of *d*-limonene:butylated hydoxyanisol mixture (Citrisolv™, Fisher Scientific, Pittsburgh, PA), washed in ethanol, and hydrated with a series of descending concentrations of ethanol in water. After washing the sections were quenched with 3% hydrogen peroxide in PBS to remove endogenous peroxidases and incubated with equilibration buffer and later with a reaction mixture containing digoxygenin-dUTP and terminal deoxynucleotidyl transferase (TdT) enzyme (Oncor). The sections were washed and treated with diluted anti-digoxygenin antibody conjugated with peroxidase. The localized peroxidase enzyme was visualized with diaminobenzidene yielding a brown product. The sections were lightly counter-stained with hematoxylin and observed under a light microscope. Positive (rat testis) and negative controls (sections without the reaction mixture) were simultaneously assayed. In each section, the apoptotic cells were counted and the number was then normalized to a unit area as previously described [8].

3.4. Enumeration of proliferating cells in liver

Paraffin-fixed liver tissues were sectioned at 5 μm, mounted on glass slides and deparaffinated with several changes of Citrosolv™ and routinely hydrated with descending serial dilutions of ethanol. Antigens were retrieved by heat treatment in a steamer containing citrate buffer (pH 6.5) for 25 min. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide and sections treated to block non-specific binding with a solution containing goat serum and avidin–biotin blocking kit (Vector Laboratories, Burlingame, CA). The sections were treated overnight at 4 °C with 1 : 100 dilution of PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary biotinylated antibody was applied and sections stained with Vectastain ABC® kit as per manufacturer's protocol (Vector Laboratories). Tissues were visually examined under a light microscope and the number of PCNA-positive cells counted in the section and normalized to the unit area as described previously [8].

3.5. RNA isolation and ribonuclease protection assay

Total RNA was isolated from frozen livers (ca. 70–80 mg) with TRI® reagent (Molecular Research Center, Cincinnati, OH), using a protocol described earlier [19]. Frozen tissues were ground in liquid nitrogen in a mortar, TRI® reagent added and tissues further homogenized. The RPA was performed on samples using RiboQuant™ RPA kits (Pharmingen, San Diego, CA) [14]. Briefly, three template sets, one for CD95 signaling molecules (*CD95*, *CD95L*, *FADD*, *FAP*, *FAF*, *TRAIL*, *TRADD*, *RIP*, *TNFR1* and *caspase 8*), another for cytokines (*IL-1α*, *IL-1β*, *IL-6*, *IL-10*, *IL-12p35*, *IL-12p40*, *IFNγ*, *IL-1Ra*, and *migration inhibitory factor* [*MIF*]), and a third mouse template set containing other apoptosis-related genes (*b-myc*, *c-myc*, *max*, *mad*, *bcl-2*, *bax*, *bad*, *caspase 3* and *caspase 6*), were used. Each set had *L32* and *GAPDH* as internal controls. The synthesis of high specific-activity [α^{32}P]UTP-labeled T7 RNA polymerase-directed anti-sense RNA probes was done using the in vitro transcription kit according to manufacturer's protocols (Pharmingen). An aliquot of 50 μg of sample RNA was hybridized with the probe overnight and digested with RNase A and RNase T1. The RNase-protected probes were purified and resolved on a denaturing polyacrylamide gel. The gels with [α^{32}P]-labeled bands were exposed to an FX Imaging Screen K-HD® (Bio-Rad Laboratories, Hercules, CA) for 6–16 h and scanned by Bio-Rad Molecular Imager® FX. The relative gene expression was digitized using Quantity One® software provided by the manufacturer (Bio-Rad) and normalized against the house keeping gene, ribosomal protein *L32*. The data were identical when normalized against *GAPDH*. Signal for some of the factors, e.g., *IL-6*,

IL-10, *IL-12p35*, *IL-12p40*, *IFNγ*, was too weak to provide meaningful data and has not been reported.

3.6. Statistics

Data from these studies were analyzed by a single-factor analysis of variance (ANOVA) followed by a post hoc Duncan's multiple-range test. Hepatic apoptosis and proliferating cell counts were compared by means of the Wilcoxon Rank Sum Test (nonparametric comparison). All tests were performed using an SAS computer program (SAS Institute, Cary, NC). The level of $p \leq 0.05$ was considered statistically significant; lower p values are indicated with respective results.

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References

[1] Batey RG, Wang J. Molecular pathogenesis of T lymphocyte-induced liver injury in alcoholic hepatitis. Front Biosci 2002;7:d1662–d1675.
[2] Blazka ME, Wilmer JL, Holladay SD, Wilson RE, Luster MI. Role of proinflammatory cytokines in acetaminophen hepatotoxicity. Toxicol Appl Pharmacol 1995;133:43–52.
[3] Czaja MJ, Xu J, Alt E. Prevention of carbon tetrachloride-induced rat-liver injury by soluble tumor-necrosis-factor receptor. Gastroenterology 1995;108:1849–54.
[4] Horn TL, Bhattacharjee A, Schook LB, Rutherford MS. Altered hepatic mRNA expression of apoptotic genes during dimethylnitrosamine exposure. Toxicol Sci 2000;57:240–9.
[5] Kayama F, Yoshida T, Elwell MR, Luster MI. Role of tumor necrosis factor-alpha in cadmium-induced hepatotoxicity. Toxicol Appl Pharmacol 1995;131:224–34.
[6] Ksontini R, Colagiovanni DB, Josephs MD, Edwards CK III, Tannahill CL, Solorzano CC, et al. Disparate roles for TNF-α and Fas ligand in concanavalin A-induced hepatitis. J Immunol 1998;160:4082–9.
[7] Ogasawara K, Takeda K, Hashimoto W, Satoh M, Okuyama R, Yanai N, et al. Involvement of NK1+ T cells and their IFN-γ production in the generalized Shwartzman reaction. J Immunol 1998;160:3522–7.
[8] Sharma RP, Dugyala RR, Voss KA. Demonstration of in-situ apoptosis in mouse liver and kidney after short-term repeated exposure to fumonisin B_1. J Comp Pathol 1997;117:371–81.
[9] Marasas WF. Discovery and occurrence of the fumonisins: a historical perspective. Environ Health Perspect 2001;109(Suppl 2): 239–43.
[10] Howard PC, Eppley RM, Stack ME, Warbritton A, Voss KA, Lorentzen RJ, et al. Fumonisin B_1 carcinogenicity in a two-year feeding study using F344 rats and B6C3F1 mice. Environ Health Perspect 2001;109(Suppl 2):277–82.
[11] Ueno Y, Iijima K, Wang SD, Sugiura Y, Sekijima M, Tanaka T, et al. Fumonisins as a possible contributory risk factor for primary liver cancer: a 3-year study of corn harvested in Haimen, China, by HPLC and ELISA. Food Chem Toxicol 1997;35: 1143–50.
[12] Tsunoda M, Sharma RP, Riley RT. Early fumonisin B_1 toxicity in relation to disrupted sphingolipid metabolism in male BALB/c mice. J Biochem Mol Toxicol 1998;12:281–9.
[13] Riley RT, Enongene E, Voss KA, Norred WP, Meredith FI, Sharma RP, et al. Sphingolipid perturbations as mechanisms for fumonisin carcinogenesis. Environ Health Perspect 2001; 109(Suppl 2):301–8.
[14] Bhandari N, Brown CC, Sharma RP. Fumonisin B_1-induced localized activation of cytokine network in mouse liver. Food Chem Toxicol 2002;40:1483–91.
[15] Bhandari N, Sharma RP. Modulation of selected cell signaling genes in mouse liver by fumonisin B_1. Chem Biol Interact 2002;139:317–31.
[16] Bhandari N, Sharma RP. Fumonisin B_1-induced alterations in cytokine expression and apoptosis signaling genes in mouse liver and kidney after an acute exposure. Toxicology 2002;172: 81–92.
[17] Dugyala RR, Sharma RP, Tsunoda M, Riley RT. Tumor necrosis factor-α as a contributor in fumonisin B_1 toxicity. J Pharmacol Exp Ther 1998;285:317–24.
[18] Sharma RP, Bhandari N, He Q, Riley RT, Voss KA. Decreased fumonisin hepatotoxicity in mice with a targeted deletion of tumor necrosis factor receptor 1. Toxicology 2001;159:69–79.
[19] Sharma RP, Bhandari N, Riley RT, Voss KA, Meredith FI. Tolerance to fumonisin toxicity in a mouse strain lacking the P75 tumor necrosis factor receptor. Toxicology 2000;143: 183–94.
[20] Sharma RP, He Q, Meredith FI, Riley RT, Voss KA. Paradoxical role of tumor necrosis factor α in fumonisin-induced hepatotoxicity in mice. Toxicology 2002;180:221.
[21] Tanaka Y, Takahashi A, Watanabe K, Takayama K, Yahata T, Habu S, et al. A pivotal role of IL-12 in Th1-dependent mouse liver injury. Int Immunol 1996;8:569–76.
[22] Galle PR, Hofmann WJ, Walczak H, Schaller H, Otto G, Stremmel W, et al. Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. J Exp Med 1995;182: 1223–30.
[23] MacEwan DJ. TNF receptor subtype signalling: differences and cellular consequences. Cell Signal 2002;14:477–92.
[24] Crowe PD, Vanarsdale TL, Walter BN, Ware CF, Hession C, Ehrenfels B, et al. A lymphotoxin-β-specific receptor. Science 1994;264:707–10.
[25] Pasparakis M, Alexopoulou L, Episkopou V, Kollias G. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF α in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J Exp Med 1996;184:1397–411.
[26] Pasparakis M, Kousteni S, Peschon J, Kollias G. Tumor necrosis factor and the p55TNF receptor are required for optimal development of the marginal sinus and for migration of follicular dendritic cell precursors into splenic follicles. Cell Immunol 2000;201:33–41.
[27] Marino MW, Dunn A, Grail D, Inglese M, Noguchi Y, Richards E, et al. Characterization of tumor necrosis factor-deficient mice. Proc Natl Acad Sci U S A 1997;94:8093–8.
[28] Kaneko H, Yamada H, Mizuno S, Udagawa T, Kazumi Y, Sekikawa K, et al. Role of tumor necrosis factor-alpha in mycobacterium-induced granuloma formation in tumor necrosis factor-α-deficient mice. Lab Invest 1999;79:379–86.

[29] Condo I, Testi R. Intracellular mediators of programmed cell death initiated at the cell surface receptor Fas. Transpl Int 2000;13(Suppl 1):S3–S6.
[30] Magnusson C, Vaux DL. Signalling by CD95 and TNF receptors: not only life and death. Immunol Cell Biol 1999;77:41–6.
[31] Srivastava RK. TRAIL/Apo-2L: mechanisms and clinical applications in cancer. Neoplasia 2001;3:535–46.
[32] French LE, Hahne M, Viard I, Radlgruber G, Zanone R, Becker K, et al. Fas and Fas ligand in embryos and adult mice: ligand expression in several immune-privileged tissues and coexpression in adult tissues characterized by apoptotic cell turnover. J Cell Biol 1996;133:335–43.
[33] Ni R, Tomita Y, Matsuda K, Ichihara A, Ishimura K, Ogasawara J, et al. Fas-mediated apoptosis in primary cultured mouse hepatocytes. Exp Cell Res 1994;215:332–7.
[34] Leithauser F, Dhein J, Mechtersheimer G, Koretz K, Bruderlein S, Henne C, et al. Constitutive and induced expression of APO-1, a new member of the nerve growth factor/tumor necrosis factor receptor superfamily, in normal and neoplastic cells. Lab Invest 1993;69:415–29.
[35] Takii Y, Hashimoto S, Iiai T, Watanabe H, Hatakeyama K, Abo T. Increase in the proportion of granulated CD56+ T cells in patients with malignancy. Clin Exp Immunol 1994; 97:522–7.
[36] Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 2000;1:489–95.
[37] Gores GJ, Kaufmann SH. Is TRAIL hepatotoxic? Hepatology 2001;34:3–6.
[38] Wu GS, Burns TF, Zhan Y, Alnemri ES, el Deiry WS. Molecular cloning and functional analysis of the mouse homologue of the KILLER/DR5 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor. Cancer Res 1999;59: 2770–5.
[39] Walczak H, Krammer PH. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res 2000;256:58–66.
[40] Baudino TA, Cleveland JL. The Max network gone mad. Mol Cell Biol 2001;21:691–702.
[41] Dang CV. c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol 1999;19:1–11.
[42] Degenhardt K, Sundararajan R, Lindsten T, Thompson C, White E. Bax and Bak independently promote cytochrome *C* release from mitochondria. J Biol Chem 2002;277:14127–34.
[43] Ungefroren H, Kruse ML, Trauzold A, Roeschmann S, Roeder C, Arlt A, et al. FAP-1 in pancreatic cancer cells: functional and mechanistic studies on its inhibitory role in CD95-mediated apoptosis. J Cell Sci 2001;114:2735–46.
[44] Arend W. The balance between IL-1 and IL-1Ra in disease. Cytokine Growth Factor Rev 2002;13:323.
[45] Gennero I, Fauvel J, Nieto M, Cariven C, Gaits F, Briand-Mesange F, et al. Apoptotic effect of sphingosine 1-phosphate and increased sphingosine 1-phosphate hydrolysis on mesangial cells cultured at low cell density. J Biol Chem 2002;277: 12724–34.
[46] Bondy GS, Barker MG, Lombaert GA, Armstrong CL, Fernie SM, Gurofsky S, et al. A comparison of clinical, histopathological and cell-cycle markers in rats receiving the fungal toxins fumonisin B_1 or fumonisin B_2 by intraperitoneal injection. Food Chem Toxicol 2000;38:873–86.
[47] Jones C, Ciacci-Zanella JR, Zhang Y, Henderson G, Dickman M. Analysis of fumonisin B_1-induced apoptosis. Environ Health Perspect 2001;109(Suppl 2):315–20.
[48] Meredith FI, Bacon CW, Plattner RD, Norred WP. Preparative LC isolation and purification of fumonisin B_1 from rice cultures. J Agric Food Chem 1996;44:195–8.